

## Appendix K

### Brief Overviews of Assays Considered for Tier 1 Screening

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This Appendix contains judgments about the utility and level of “validation” of specific assays that were discussed in varying levels of detail by the Screening and Testing Work Group (STWG) members. The summaries should not be considered “consensus” materials, but rather submissions, from various members of the STWG, developed to assist the full group in arriving at their final set of recommendations, which were subsequently forwarded to the full Endocrine Disruptor Screening and Testing Advisory Committee for further discussion.

## **Estrogen and Anti-estrogen - Intrinsic Activity**

### **I.**

#### **A. Rat (and other non-human mammalian and avian) ER Binding Assay**

##### **DESCRIPTION**

*In vitro* affinity of toxicants for rER.

##### **DEGREE OF USE**

Extensive, for 20 years, largest *in vitro* database, fairly easy *in vitro* assay.

##### **DURATION**

24 hours.

##### **ASSAY STABILITY**

Good at low temperatures, receptor degradation at higher temperatures.

##### **DOES IT METABOLIZE TOXICANTS**

No

##### **ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED**

Yes

##### **ARE SPECIAL SKILLS/TRAINING NEEDED**

Yes, but can be trained in short time.

##### **HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY**

It could easily be standardized, most labs run it at 4° C for 18 hours.

##### **SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS**

Very sensitive for high to moderate affinity ligands, can be run over a wide range of log doses.

##### **ARE THERE KNOWN FALSE POSITIVES**

To the degree that it and all other *in vitro* assays cannot account for ADME, things that bind ER may not be active *in vivo*. Other than this, no false positives.

##### **OR FALSE NEGATIVES**

Yes, if assay is conducted at low temp, which is generally true, some things fail to bind ER under these conditions, but work *in vivo* and activate ER in proliferation assays and transfected cells (and effect can be blocked with anti-estrogen). In other cases, solubility at low temperature of lipophilic toxicants may preclude detection.

##### **SPECIFICITY-ARE THERE OTHER MECHANISMS TO PRODUCE THIS EFFECT (FALSE POSITIVES)**

No

##### **COMMENTS**

Both agonists and antagonists bind ER, good for screening but additional information is required to determine if it is estrogenic or anti-estrogenic *in vitro*.

Requires radioactivity. Toxicant solubility and degradation are a problem in this and all other *in vitro* assays. GLP requirements should be established to verify culture conditions to the same degree that we verify dosing solutions for *in vivo* tests.

**B. hER Binding From MCF-7 Cell Lysate****DESCRIPTION**

Competitive binding of toxicants to hER in human cell lysate.

**DEGREE OF USE**

Widespread since 1973.

**DURATION**

A few days.

**COMMENTS**

Problems similar to rat or other mammalian RBA assays for ER.

**C. Estrogen Competitor (Binding) Screening Assay (A Receptor/Ligand Assay, PanVera)****DESCRIPTION**

The assay utilizes recombinant, human estrogen receptor and an autofluorescent, high affinity estrogen ligand. Competitors are identified by their ability to disrupt binding of the ER and fluorescent estrogen. Binding is quantified by fluorescence polarization. This technique allows for the direct measurement of the bound to free ratio of the ligand at equilibrium, in solution, with no precipitation, dialysis, extraction, or any other separation of bound and free ligand required.

**DEGREE OF USE**

This assay is currently in use in drug discovery as both a primary screen to search for new estrogen ligands and as a secondary screen to characterize lead compounds.

**DURATION**

Receptor/ligand equilibrium is the rate limiting step. Including a one-hour room temperature equilibration, it should take about two hours from sample prep to measurement of polarization.

**ASSAY STABILITY**

Recombinant ER is stable at room temperature for at least six hours.

**DOES IT METABOLIZE TOXICANTS**

No

**ARE SPECIAL EQUIPMENT REQUIREMENTS MANDATED**

A fluorescence polarimeter is required (\$18.5K for single tube instrument, \$30K for 96- to 384-well instrument).

**ARE SPECIAL SKILLS/TRAINING NEEDED**

No, only basic lab skills and GLP training is needed.

**HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY**

Yes, typically use estradiol as a standard for IC<sub>50</sub> values comparison. Assay has been used to determine IC<sub>50</sub> values and K<sub>i</sub> values for tamoxifen, estradiol, estrone, estriol, estrone, chlordane, and others. Because assay is at true equilibrium, K<sub>i</sub> values can be calculated.

**SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS**

Similar to other receptor/ligand binding assays. Sensitivity dependent not on assay, but on solubility of the test compound. For example, to determine an IC<sub>50</sub> value of 50 mM, the test compound would have to be soluble in the test matrix at least 50mM.

**ARE THERE KNOWN FALSE POSITIVES**

As with other receptor binding assays, non-competitive inhibition is possible, but rare.

**OR FALSE NEGATIVES**

Probably less than receptor/ligand assays performed at lower temperatures. This assay can be performed at 4° to 37° C.

**SPECIFICITY-ARE THERE OTHER MECHANISMS TO PRODUCE THIS EFFECT (FALSE POSITIVES)**

No

**COMMENTS**

Purified receptor is well characterized and therefore activity is more reproducible than lysates. No radioactivity. Assay is performed at true equilibrium. Technique is non-destructive. Reactions in disposable tubes can be remeasured under various conditions. For example, competition curve can be measured at 4° C, incubated at 20° C and then measured at 20° C. Assay can be performed in a multi-well format and automated. Technique should be applicable to multiple species ER to determine relative potency in those species. Assay is very simple: (1) add ER and fluorescent estrogen to test compound, (2) incubate, and (3) measure polarization.

**Estrogen and Anti-estrogen - *In Vitro*****II.****A. MCF-7 Proliferation Assay (ESCREEN)****DESCRIPTION**

Measures growth of cells *in vitro* in response to ER modulators.

**DEGREE OF USE**

Thousands of chemicals studied, several toxicants.

**DURATION**

One rep takes a week

**ASSAY STABILITY**

Varies with serum batch, MCF-7 cell subclone, passage number, etc.

**DOES IT METABOLIZE TOXICANTS**

Relatively unknown, has aromatase activity, but much less than kidney or liver cells. Reports about metabolic activation of methoxychlor have not been verified.

**ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED**

Yes, cell culture and cell counting equipment.

**ARE SPECIAL SKILLS/TRAINING NEEDED**

Yes, cells must be maintained the same, week after week.

**HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY**

Has not been standardized. Some factors could be (subclone type), while others (serum variability) would be difficult. Competent labs appear to get similar qualitative, if not quantitative results.

**SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS**

Very sensitive, one of the most sensitive assays.

**ARE THERE KNOWN FALSE POSITIVES**

Yes, some growth factors, other steroids may TCDD, etc., can influence proliferation assay, or kill cells or make them “sick” (would appear anti-estrogenic).

**OR FALSE NEGATIVES**

Things that require metabolic activation may be missed in this and all other *in vitro* assays.

**SPECIFICITY-ARE THERE OTHER MECHANISMS TO PRODUCE THIS EFFECT (FALSE POSITIVES)**

As above, this is a complex response of an unknown number of genes that can be influenced by other mechanisms. Could also be false negatives if something binds ER and activates different genes.

**COMMENTS.**

Best assay of the ‘70s and ‘80s. Controls needed for cell viability, health and run with anti-estrogen to reverse effect in order to confirm that proliferation was mediated via ER.

**B. YES-Yeast Estrogen Screen****DESCRIPTION**

*In vitro* assay using yeast cells transformed with hER (whole or fragment), VIT promoter and reporter (luc or CAT) construct.

**DEGREE OF USE**

Widespread in industry for drugs, a handful of papers on use with toxicants. Results quite mixed.

**DURATION**

Short-term, 24 hours.

**ASSAY STABILITY**

Response varies greatly from subclone to subclone for certain types of xenoestrogens. Yeast have a cell wall and some strains have transport systems that render them drug resistant (i.e. dexamethasone). Other strains even appear to transport estradiol out of the cell. Response varies greatly, depending upon the type of hER gene construct (whole versus fragment).

**DOES IT METABOLIZE TOXICANTS**

Unknown

**ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED**

Cell culture equipment and techniques.

**ARE SPECIAL SKILLS/TRAINING NEEDED**

Permanently transformed so may be easier than transient transfections, but *in vitro* training required.

**HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY**

Has not been standardized at present. Standardization could be achieved if the “best” subclone and gene/reporter construct could be determined. May be premature to standardize without further development as a research tool.

**SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS**

Good for some (alkylphenols), some strains are very insensitive to chlorinated pesticides.

ARE THERE KNOWN FALSE POSITIVES OR FALSE NEGATIVE

Depends upon specificity of reporter construct, but as good as any ER-binding assay. Several false negatives noted for some strains.

SPECIFICITY-ARE THERE OTHER MECHANISMS TO PRODUCE THIS EFFECT (FALSE POSITIVES)

Depends on gene construct employed.

COMMENTS

As employed by some, is more like a binding assay because it fails to discriminate between agonist and antagonists. Given limitations of transport, is a curious choice given the minimal information provided and high potential false negatives.

**C. MVLN Assay. Stably Transfected Reporter Gene Assay in Mammalian Cells**

DESCRIPTION

The assay utilizes a mammalian cell line (MCF-7 with endogenous human ER) that has been stably transfected with an ER specific reporter gene (Vit-Luc).

DEGREE OF USE

Used in various labs for pharmaceutical and environmental research.

DURATION

From plating cells to harvesting lysate and Luc activity takes two to three days.

ASSAY STABILITY

Stably transfected cells maintain same level of activity for at least 30 passages. Activity is maintained after typical cell culture freezing storage cycles.

DOSE IT METABOLIZE TOXICANTS

May have aromatase and other limited metabolic capacities of other MCF-7 subclones.

ARE SPECIAL EQUIPMENT REQUIREMENTS MANDATED

Typical cell culture equipment as well as a luminometer to measure Luc activity.

ARE SPECIAL SKILLS/TRAINING NEEDED

Tissue culture, basic lab skills GLP training is needed.

HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY

Yes, activity for various steroids and nonsteroids are comparable to other transient reporter assays. Stably transfected cells can be easily distributed.

SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE COMPOUNDS

Highly sensitive. EC50 for E2 in the pM range.

ARE THERE KNOWN FALSE POSITIVES

No, hypothetically ER could be activated by phosphorylation pathways.

OR FALSE NEGATIVES

No, would expect only very specific activation of ER.

SPECIFICALLY - ARE THERE OTHER MECHANISMS TO PRODUCE THE EFFECT (FALSE POSITIVES)

Hypothetically ER could be activated by phosphorylation pathways.

COMMENTS

Specific for ER transcription activation. Done in mammalian cells. Utilizes human ER. Easy assay, no transfections. High assay signal due to all cells expressing reporter. Can be done in dishes, 12, 24 or 96 well plates. Can be automated.

**D. Cotransfected Reporter Gene Assay in Mammalian Cells (e.g., CV-1 or COS Cells)**  
DESCRIPTION

The assay utilizes a mammalian cell line (CV-1, COS) that has been transiently transfected with ER as well as an ER specific reporter gene (Vit-Luc).

DEGREE OF USE

Used widely in various labs for pharmaceutical and environmental research.

DURATION

From plating cells to harvesting lysate and Luc activity determination takes two to three days.

ASSAY STABILITY

Stability depends on transfection efficiency between experiments.

DOSE IT METABOLIZE TOXICANTS

May have limited metabolic capacities.

ARE SPECIAL EQUIPMENT REQUIREMENTS MANDATED

Typical cell culture equipment as well as a luminometer to measure Luc activity.

ARE SPECIAL SKILLS/TRAINING NEEDED

Tissue culture, transfection, basic lab skills and GLP training is needed.

HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY

Yes, when standardized relative to % activity of E2, results are comparable from assay to assay.

SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE COMPOUNDS

Sensitive. EC50 for E2 in the 10pM range.

ARE THERE KNOWN FALSE POSITIVES

No, hypothetically ER could be activated by phosphorylation pathways.

OR FALSE NEGATIVES

No, would expect only very specific activation of ER.

SPECIFICALLY - ARE THERE OTHER MECHANISMS TO PRODUCE THE EFFECT (FALSE POSITIVES)

Hypothetically ER could be activated by phosphorylation pathways.

COMMENTS

Specific for ER transcription activation. Done in mammalian cells. Can utilize ER from any cloned source (any species). Requires transfections. Can be done in dishes or 12 well plates. Can transfect in different reporter constructs to compare ligand selective gene regulation.

**E. Stably Transfected Reporter Gene Assay in Mammalian Cells (e.g., MCF-7 Cells)**  
DESCRIPTION

The assay utilizes a mammalian cell line (MCF-7 with endogenous human ER) that has been stably transfected with an ER specific reporter gene (Vit-Luc).

**DEGREE OF USE**

Used in various labs for pharmaceutical and environmental research.

**DURATION**

From plating cells to harvesting lysate and Luc activity determination takes two to three days.

**ASSAY STABILITY**

Stably transfected cells maintain same level of activity for at least 30 passages. Activity is maintained after typical cell culture freezing storage cycles.

**DOSE IT METABOLIZE TOXICANTS**

May have aromatase and other limited metabolic capacities of other MCF-7 subclones.

**ARE SPECIAL EQUIPMENT REQUIREMENTS MANDATED**

Typical cell culture equipment as well as a luminometer to measure Luc activity.

**ARE SPECIAL SKILLS/TRAINING NEEDED**

Tissue culture, basic lab skills and GLP training is needed.

**HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY**

Yes, activity for various steroids and nonsteroids are comparable to other transient reporter assays. Stably transfected cells can be easily distributed.

**SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE COMPOUNDS**

Highly sensitive. EC50 for E2 in the pM range.

**ARE THERE KNOWN FALSE POSITIVES**

No, hypothetically ER could be activated by phosphorylation pathways.

**OR FALSE NEGATIVES**

No, would expect only very specific activation of ER.

**SPECIFICALLY - ARE THERE OTHER MECHANISMS TO PRODUCE THE EFFECT (FALSE POSITIVES)**

Hypothetically ER could be activated by phosphorylation pathways.

**COMMENTS**

Specific for ER transcription activation. Done in mammalian cells. Utilizes human ER. Easy assay, no transfection. High assay signal due to all cells expressing reporter. Can be done in dishes, 12, 24 or 96 well plates. Can be automated.

**Estrogen and Anti-estrogen - *In Vivo*****III.****A. Uterine Peroxidase Assay****COMMENTS**

Uterine peroxidase activity is thought to be estrogen regulated, therefore monitoring its levels may serve as a means to determine the estrogenicity of chemicals. Johri et al. used this method to determine estrogenic/anti-estrogenic potential of anti-fertility substances. In this assay Charles Foster rats were given either oral doses ranging from 1.5-10.0 mg/kg or subcutaneous injections (e.g. estradiol) of 0.1-1.0 mg/rat/day. After 3 days (or longer) of dosing the animals were sacrificed 24 hours after the last dose. Uteri were excised and homogenized in sodium acetate. Peroxidase activity was determined (by the Alexander Method) and reaction rates were monitored and enzyme activity expressed as D A353/mg protein/min.

**B. Developmental Uterotrophic Assay****DESCRIPTION**

Rat; treatment on postnatal days (pnd) 10-14 with a variety of estrogens/antiestrogens inhibits uterine gland appearance and increases uterine weight and luminal epithelial height measured on pnd 14 or in adults. D-R curves; replicated in mice.

**ARE SPECIAL EQUIPMENT REQUIREMENTS MANDATED**

Balance; ocular micrometer; standard histology equipment for H&E staining of sections.

**ARE SPECIAL SKILLS/TRAINING NEEDED**

Animal handling and dosing; tissue removal and weighing; slide preparation including vertical placement of uteri in paraffin; simple microscopy.

**AVAILABILITY**

Available in open literature.

**HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY**

Standardization; use of positive controls; stable values for control and max responses over ten years and ~ ten estrogens.

**SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS**

Sensitivity: DES, EE2~1ug/Kg/day ED50 for luminal epithelial height (3x increase max) or for doubling uterine weight, ~0.3 ug/Kg/day ED50 for gland inhibition (~4 glands/section inhibited down to 0 glands/section.

**SPECIFICITY-ARE THERE OTHER MECHANISMS TO PRODUCE THIS EFFECT (FALSE POSITIVES)**

Estrogens and antiestrogens; need to examine other hormones to establish specificity.

**COMMENTS**

General and specific suggestions today for the estrogens/antiestrogens. General: (1) Use a pure antiestrogen such as ICI 182,780 to confirm specificity of agonist/antagonist activity *in vitro/in vivo*?; (2) All tasks- should we eventually consider standardizing assays so data can be compared/modeled and quality control checked?, include positive/negative controls?; (3) Are we assuming multipoint assays or (hopefully not) single point assays?; (4) Where there are choices sensitivity should be considered; all other things being equal a chemical with, for example, low solubility, would be more likely to be detected in the more sensitive assay, what should the lower limits be on the hormonal activity relative to a standard such as E2?; (5) Do cell assays need cytotoxicity measures to help interpretation of data?

**Specific:**

For *in vivo* detection of estrogenicity/antiestrogenicity/steroidogenesis effects, consider the following. On postnatal days (PND) 10-14, the rat ovary actively makes estrogens which increase uterine weight (normalized to body weight) by 30% on PND14. After about PND 16, estrogen levels drop to the lower but still detectable values found in immature rats. Ovariectomy or ICI 182,780 reduces uterine weight to control or lower values on PND 14 or beyond. Treatment with agonists on PND 10-14 increases uterine weight several fold, increases the height of the luminal epithelium (LEH) three-fold and inhibits the appearance of uterine glands. Glands begin to appear on PND 9 by

invagination from the luminal epithelium and the process is over by PND 16-17. The later two measures are done on cross-sections of paraffin embedded uteri by ocular micrometer or image analysis for LEH and gland counts per section. Gland numbers are 4-5 in controls and decrease to almost zero from agonist treatment. So agonists increase uterine weight and LEH and decrease gland numbers. The triphenylethylene partial agonists/partial antagonists such as tamoxifen, clomiphene, toremifene, etc. show marginal uterine weight gain, increase LEH three-fold and inhibit gland genesis. This pattern contrasts with the complete agonists. ICI 182,780, a complete antagonist, reduces uterine weight but has no effect on gland genesis or LEH, a different pattern than those above. Important here is that the patterns of responses in a single experiment distinguishes these pharmacological classes rather than depending on sequential experiments each with a different design. Additionally, one endpoint, gland genesis, is a classical developmental toxicity endpoint; there is a defined ontogenic pattern, a sensitive period, and an adverse outcome (gland numbers are reduced or absent in adults following PND10-14 treatment). We don't have experience with other mixed agonists/antagonists with the possible exception of some phytoestrogens for which the verdict is not yet in. An additional, but untested feature, is that chemicals which interfere with steroidogenesis should be active in this system; chemicals which increase steroidogenesis will act like an agonist but fail to bind to the ER, while those that decrease steroidogenesis will act like a pure antagonist (or ovariectomy) but fail to bind to the ER. Any nominations for chemicals in this category for us to look at? Virtues of the assay are that there are an average of six female pups per dam which can be randomized to different dams versus waiting for animals to mature or the need for ovariectomy and waiting for ten days; the pups stay with dams in a single cage throughout the experiment. Arrival of dams on GD 2 and completion of the study on PND 14 results in an average of five to six cage days per animal, while providing multiple endpoints and the ability to distinguish the pharmacological activity, and includes an endpoint for developmental toxicity. These features should be compared to the animal numbers, cage costs, personnel time and length of time to conduct the sequential experiments as suggested in the outline.

#### Comments on metabolism:

Because only the *in vivo* assays have the potential to detect prohormone metabolism to an active hormone, this property should be included in the rationale for the Tier 1 battery including an *in vivo* component.

#### Comments on use of cell constructs/cultures:

These, along with the ER binding should, if positive, be seen as providing high priority for moving into Tier 1 and Tier 2 *in vivo* tests. Likewise, chemicals in the prescreens that are "positive" should be moved immediately into the tiers. This sequence is differentiated from one that would move a group of chemicals lockstep through the prescreen and Tier 1 steps with no priority (urgency) to move to higher tiers. With lockstep testing, market and regulatory decisions on chemicals that are a problem would ultimately be delayed to some unknown extent while exposure continues. In particular, the *in vivo* assays could be seen as inducing significant delays.

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### C. Uterine Weight Bioassay in Juvenile or Adult Ovariectomized Female Rats

#### DESCRIPTION

One to three oral or injected doses of toxicant to immature female rat (18-21 d), longer in adult ovx'ed female.

#### DEGREE OF USE

One of original "gold" standards for screening for estrogenicity, used for about 80 years.  
Extensive database on toxicants.

#### DURATION

One to three days, or weeks in adult.

#### ASSAY STABILITY

Very stable unless juvenile females older than 24 days at necropsy, or sloppily designed at weaning.

#### DOES IT METABOLIZE TOXICANTS

Yes

ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED

Good balance and scissors.

ARE SPECIAL SKILLS/TRAINING NEEDED

Necropsy skills.

HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY

Standardized to some degree. Could be improved (trim fat, weigh with and without fluid).

SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS

One of the most sensitive *in vivo* short-term assays using immature or adult females. (Rank #1). Nonylphenol, octylphenol, bisphenol A, methoxychlor, estradiol 17 I, kepone, etc. are positive.

ARE THERE KNOWN FALSE POSITIVES

Yes, intact female is used, hence, effects on hypothalamic-pit axis, and GH or prolactin alterations could affect this process. In addition, aromatizable and nonaromatizable (via AR) can affect weight. Even a few false positives in ovariectomized adult females.

OR FALSE-NEGATIVES

Some potential, as some chemicals are poorly absorbed interaperitoneal and are more effective when given orally, while many others are less effective after oral administration. As with any *in vivo* assay, chemicals that are estrogenic in binding or cell assays may be negative, albeit not falsely so, if they are not absorbed, are metabolically inactivated or excreted such that the active material never reaches the targets.

SPECIFICITY-ARE THERE OTHER MECHANISMS TO PRODUCE THIS EFFECT (FALSE POSITIVES)

Yes, as indicated above.

COMMENTS

Necropsy of animals six hours after last dose, of three, is more effective than 24 hours later in detection of weak estrogens. In adult female, this endpoint can be coupled with several others.

**D. Vaginal Smears (Mucification and Cornification)**

DESCRIPTION

Noninvasive measurement of estrogenicity in intact or ovariectomized female rat. Vaginal lavages are examined for cell types.

DEGREE OF USE

Extensive use for over 80 years (Allen-Doisy Assay).

DURATION

Moderate to long duration screen requires daily examination of vaginal cells by microscopy. At least one week in duration can dose for months at low dosage levels.

ASSAY STABILITY

Very stable across and within labs.

DOES IT METABOLIZE TOXICANTS

Yes

ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED

Microscope

**ARE SPECIAL SKILLS/TRAINING NEEDED**

Not difficult, but requires more expertise than determination of vaginal opening or uterine weight. Data analysis can also be relatively complicated.

**HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY**

Not standardized with regard to a number of factors (staining versus no staining, read and discard wet sample or save dry and read later, data recording and classification, method of data analysis). In spite of the lack of standardization, this is a robust measure as I am not aware of a case where different labs, using different methods, did not get the same results. Could be easily standardized.

**SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS**

Xenoestrogen toxicant and pesticide data indicate clearly that vaginal cornification is less sensitive than a number of other screens for estrogenicity (Cluster Rank #4) below uterine weight, uterine histology, uterine biochemical measures, vaginal histology, vaginal mucification, vaginal opening, induction of mating behavior, etc. However, one data set with estrogenic PCBs reported vaginal cornification in the absence of an increase in uterine weight. Mucification of smears occurs before or at lower dosage levels than cornification.

**ARE THERE KNOWN FALSE POSITIVES**

As is the case with most of these screens, when intact animals are utilized, there are many treatments that alter vaginal cornification via nonestrogenic mechanisms. Conducting the test in an ovariectomized female enhances the specificity of the screen. Still, there are false positives, but the likelihood is greatly reduced.

**OR FALSE NEGATIVES**

Yes. Toxicants that accelerate or delay hypothalamic pituitary development, affect the reproductive tract directly, alter GH, or prolactin can alter VO in the intact juvenile female.

**SPECIFICITY-ARE THERE OTHER MECHANISMS TO PRODUCE THIS EFFECT (FALSE POSITIVES)**

Yes, especially in intact animals.

**COMMENTS**

Good assay that can be used in conjunction with several other endpoints. For example, dose ovx'ed (long-term) female for three weeks, take vaginal lavage daily, observe lordosis behavior, necropsy female and weight uterus, vaginal and do histology on tract. Could also add biochemical measures.

**E. Puberty. Age at Vaginal Opening (First Estrus, Onset of Cyclicity)****DESCRIPTION**

*In vivo* test of estrogenicity in intact juvenile female rats or mice. One major advantage over other assays is that this one can detect estrogens and antiestrogens (delayed VO). Acceleration and delay of VO, age at first estrus and onset of estrous cycles can occur after *in utero* exposure to xenoestrogens and other toxicants (TCDD-which also produces a permanent anomaly of VO).

**DEGREE OF USE**

One of original indices used to screen for estrogens 80 years ago. Included in most new multigenerational tests. Fair amount of xenoestrogen data. Methoxychlor, octylphenol, nonylphenol are all positive.

**DURATION**

Acceleration of VO takes from 2-3 days to about a week, after which the process begins to occur normally at puberty in controls.

**ASSAY STABILITY**

Strains vary slightly (except Fischer rat). Age at VO has changed considerably since the 1930's due to improvements in diet.

**DOES IT METABOLIZE TOXICANTS**

Yes

**ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED**

None

**ARE SPECIAL SKILLS/TRAINING NEEDED**

Ability to follow a simple protocol. Absolutely the easiest assay and the animals do not need to be killed. Response is relatively uniform, such that fairly small differences can be detected with modest sample sizes.

**HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY**

Yes

**SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS**

Falls into Cluster #1 or 2 for sensitivity below uterine weight, etc., but more sensitive than is vaginal cornification for most pesticides and toxic substances. Rivals uterine weight in juvenile rat for some chemicals if oral dosing is employed in uterotrophic assay.

**ARE THERE KNOWN FALSE POSITIVES**

An apical test, several mechanisms can lead to accelerated or delays in VO.

**SPECIFICITY-ARE THERE OTHER MECHANISMS TO PRODUCE THIS EFFECT (FALSE POSITIVES)**

Yes.

**COMMENTS**

Can be coupled with several other assays if dosing is continued like vaginal cornification, and for Ah-receptor, and thyroid hormone effects. If VO is delayed, ovary can be studied *ex vivo* for inhibition of steroid hormone synthesis. However, cannot be conducted along with uterotrophic assay as some have tried.

**F. Induction of Female Sex Behavior (Proceptive and Receptive Behaviors)****DESCRIPTION**

*In vivo* behavioral test of lordosis induction (lordosis quotient) provides a quantitative screen for estrogenicity.

**DEGREE OF USE**

One of original, 80 year-old, tests for estrogenicity, used less extensively at present, as compared to uterine weight or vaginal cornification. Several xenoestrogens have been tested. Methoxychlor, nonylphenol, octylphenol, bisphenol A and o,p' DDT are positive, while chlordecone is negative.

## DURATION

Three days

## ASSAY STABILITY

Stable, little variability in data from lab to lab, or block to block.

## DOES IT METABOLIZE TOXICANTS

Yes

## ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED

Yes, need reverse photoperiod (or willingness to work nights) with dim lights.

Minimal equipment requirements.

## ARE SPECIAL SKILLS/TRAINING NEEDED

Some training required, but rather simple noninvasive observation.

## HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY

Yes, most protocols are quite similar and could be easily put in standard SOP.

## SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS

One of the most sensitive assays, equivalent to, or better than (due to zero variance in controls) uterine weight and histology.

## ARE THERE KNOWN FALSE POSITIVES

None, clearly the most specific test for an estrogenic response *In Vivo*.

## OR FALSE NEGATIVES

Yes, one is known, chlordecone blocks rather than induces lordosis (likely through the effect of the progesterone receptor, or as a result of the general neurotoxicity).

## SPECIFICITY-ARE THERE OTHER MECHANISMS TO PRODUCE THIS EFFECT (FALSE POSITIVES)

None known

## COMMENTS

Can be coupled with other assays. For example, dose animal, observe sex behavior, necropsy female, weigh uterus and vagina and use tissue for histology and/or biochemical measures (i.e., ODC).

**G. Feeding Behavior. Food Consumption and Growth Rate**

## DESCRIPTION

Simple *in vivo* assay which estrogens specifically retard via CNS action.

## DEGREE OF USE

Everyone measures it, and widely recognized as a sensitive effect in the toxicology of estrogens in the male rat. Methoxychlor, nonylphenol, octylphenol are positive. Bisphenol A is likely positive.

## DURATION

Appearance of estrogen-inhibited food consumption and growth are dose-related. At high dosage levels the effects are immediate, while at lower dosage levels the effects take months to be manifest.

## ASSAY STABILITY

Stable

## DOES IT METABOLIZE TOXICANTS

Yes  
ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED  
Balances  
ARE SPECIAL SKILLS/TRAINING NEEDED  
No  
HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY  
Yes  
SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS  
Likely the most sensitive response in weanling/adult male rat but much less sensitive in intact female rat, and very good in ovariectomized female.  
ARE THERE KNOWN FALSE POSITIVES  
Obviously, this endpoint can be affected by a multiplicity of mechanisms, so although sensitive it is very nonspecific.  
SPECIFICITY-ARE THERE OTHER MECHANISMS TO PRODUCE THIS EFFECT (FALSE POSITIVES)  
Yes  
COMMENTS  
Always collected, but rarely recognized in a multigen study as an estrogenic effect. Lack of recognition of body weight as sensitive endpoint leads to serious misinterpretation of multigen data.

## **H. Estrous Cyclicity**

### **DESCRIPTION**

Repeated daily observation of vaginal smears allows for determination of alterations of estrous cyclicity in rat. Can be done in other rodents, but mouse is more variable. Hamster is more regular, but rarely used and technique is quite different from rat or mouse.

### **DEGREE OF USE**

Very widespread in reproductive physiology, required in most new multigen studies. Current database for xenobiotics modest, but growing.

### **DURATION**

An absolute minimum of 10 days is needed. Guidelines typically require 15 days or longer. More useful if animals are dosed for some time period prior to treatment rather than initiating treatment with onset of observation.

### **ASSAY STABILITY**

Some variability between females (4, 5 versus 4/5 d cyclers normal. Not unusual to see 6-7 day cycles right after puberty). Some strain variability, possibly seasonal (hypothesized, but not proven), and social influences (especially in mouse).

### **DOES IT METABOLIZE TOXICANTS**

Yes

### **ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED**

Microscope

### **ARE SPECIAL SKILLS/TRAINING NEEDED**

Collecting and evaluating smears takes a little practice, data analysis is more difficult.

**HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY**

Methods of smear collection, staining (if any), preservation of sample (not required by EPA, if any) and methods of recording data and analyzing data vary from investigator to investigator. However, competent labs usually get similar results despite variable methodologies.

**SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS**

With exposure to adult or juvenile female, this assay is less sensitive to xenoestrogens than: (1) uterine weight and histological behavior; (2) vaginal opening; and (3) vaginal mucification and cornification. Less sensitive than ovarian measures to alterations of steroid hormone synthesis and ovarian morphology, due to compensation within ovary. Sensitive to disruption by hypothalamic-pituitary endocrine alterations (i.e., atrazine) of LH, FSH, GnRH, or prolactin.

In developmental studies, loss of estrous cyclicity is a sensitive response to perinatal xenoestrogen exposure via CNS defeminization. Appearance of anovulation is dose-related, can take six to nine months to appear. Too long for "screening", but all xenoestrogens that have been studied produce this affect.

**ARE THERE KNOWN FALSE POSITIVES**

As indicated above, this is an apical measure that responds to many mechanisms of action besides estrogenicity.

**OR FALSE NEGATIVES**

Weak estrogens may produce many other effects without altering estrous cyclicity.

**SPECIFICITY-ARE THERE OTHER MECHANISMS TO PRODUCE THIS EFFECT (FALSE POSITIVES)**

Estrogens, androgens, steroid hormone synthesis inhibitors and toxicants that alter LH, FSH, GnRH, or prolactin.

**COMMENTS**

Good apical test, but unfortunately less sensitive by about ten-fold to xenoestrogens (i.e. methoxychlor).

**I. Super Apical Developmental Toxicity Test****DESCRIPTION**

Expose pregnant/lactating dam and examine hormone (AR, ER, SIS, Ah, and T3) sensitive endpoints in progeny up to puberty.

**DEGREE OF USE**

Such a protocol has been used at EPA, CIIT, NIEHS for xenoestrogens, environmental antiandrogens, Ah receptor agonists, phthalates and antithyroidal toxicants (PCBs and PTU).

**DURATION**

Relatively long (two to three months) as compared to other "screens," would need to determine if it is quicker and cheaper to run this as opposed to several more focused, shorter-term assays.

**ASSAY STABILITY**

Unknown, but should be as good as any developmental test.

**DOES IT METABOLIZE TOXICANTS**

Yes

ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED

Yes, similar to needs for new multigen tests.

ARE SPECIAL SKILLS/TRAINING NEEDED

Multiplicity of *in vivo* techniques are required that are currently not used in toxicology labs, but they should be coming up to speed to implement new test guidelines.

HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY

Not standardized as used in different labs with respect to dosing or some of the assessments.  
Some standardization is now taking place between different labs.

**SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS**

Antiandrogency via AR, or SIS. In male progeny, AGD, areolas, nipples, reduced weight of prostate. Higher doses of AR-mediated, but not SIS, produce agenesis of prostate, undescended testis and agenesis of epididymis. Altered T production by testis, *ex vivo*.

Estrogenicity. Neonatal uterine weight and gland development (?), vaginal opening, age at first estrus, vaginal cornification. Prostate size in male (?), and sperm production (at high dosage levels). SIS or Antiestrogenicity. Pregnancy loss, delay in deliver by dam, delay in VO and possible altered ovarian hormone production *ex vivo*.

Antithyroid. Reduced perinatal growth and brain size. Lower serum T4 and possibly T3, elevated TSH. Also detects functional developmental alterations induced by phthalates, TCDD, etc. The following endpoints are considered to be insensitive because they take too long to assess in a screen and/or have never been detected with a pesticide or toxic substance following developmental exposure (i.e. for ER-mediated: cancer, reduced AGD, hypospadias in male of female, undescended testes or any malformations).

**ARE THERE KNOWN FALSE POSITIVES.**

Very Apical Test used to screen for several mechanisms at once.

**OR FALSE NEGATIVES.**

Not likely, if designed properly.

**IV. Anti-Estrogen - Synthesis Metabolism****A. Testis/Ovary Culture *In Vitro* or *Ex Vivo*****DESCRIPTION**

Determination of testosterone production from testicular tissue from animals treated *in vivo* (*ex vivo*) or using *in vitro* dosing. Used for EDS, estrogens, antiandrogens, several other testicular toxicants, substances that inhibit steroidogenesis. In female, minced ovary culture can be used *ex vivo* from pregnant (i.e., GD 14-16) or cycling females (proestrus for estradiol production).

**DEGREE OF USE**

A few hundred publications over the last 25 years since the advent of RIAs for testosterone. Used in other vertebrates, as well as mammals. Used by several toxicology laboratories in addition to NHEERL-EPA.

**DURATION**

One day for *in vitro*, longer for *ex vivo* (duration depends upon dosing).

**ASSAY STABILITY**

Depends on methodology.

**DOES IT METABOLIZE TOXICANTS**

Yes, for *Ex Vivo*, little or no metabolism for *in vitro*.

**ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED**

Incubator, freezer, necropsy equipment.

**ARE SPECIAL SKILLS/TRAINING NEEDED**

Some practice, but a rather simple assay that can be learned by competent technicians in a week or less. Need to be able to conduct RIAs for T, estradiol, and progesterone.

**HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY**

Has not been standardized between labs, but could be without too much difficulty.

**SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS**

Fairly sensitive.

**ARE THERE KNOWN FALSE POSITIVES**

Cell toxicants.

**OR FALSE NEGATIVES**

If metabolic activation is required then *in vitro* may not work.

**COMMENTS.**

These assays could be used rapidly to screen chemicals for ability to inhibit steroidogenesis.

**V. Androgen and Anti-androgen - Intrinsic Activity**

**A. Rat AR Equilibrium Binding Assay**

**DESCRIPTION**

*In vitro* affinity of toxicants for rAR.

**DEGREE OF USE**

Extensive, for 20 years, large *in vitro* data base, with about 20 xenoantiandrogens identified to date, easiest *in vitro* assay.

**DURATION**

24 hours

**ASSAY STABILITY**

Acceptable at low temperatures, receptor degradation at higher temps.

**DOES IT METABOLIZE TOXICANTS**

No

**ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED**

Yes

**ARE SPECIAL SKILLS/TRAINING NEEDED**

Yes, but can be trained in short time.

**HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY**

It could easily be standardized.

**SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS**

Sensitive, can be run over a wide range of log doses.

**ARE THERE KNOWN FALSE POSITIVES**

To the degree that it and all other *in vitro* assays cannot account for ADME, things that bind AR may not be active *in vivo*. Other than this, no false positives.

**OR FALSE NEGATIVES**

Yes, assay is conducted at low temp and some things fail to bind AR under these conditions, due to low solubility at low temperatures.

SPECIFICITY-ARE THERE OTHER MECHANISMS TO PRODUCE THIS EFFECT (FALSE POSITIVES)

No

COMMENTS

Both agonists and antagonists bind AR, good for screening but additional information is required to determine if it is androgenic or anti-androgenic *in vitro*. Requires radioactivity. Toxicant solubility and degradation are a problem in this and all other *in vitro* assays. GLP requirements should be established to verify incubation conditions to the same degree that we verify dosing solutions for *in vivo* tests. Should be simple, as labs are already set up to do the chemistry for *in vivo* studies.

## **B. hAR Whole Cell Binding Assay**

DESCRIPTION

*In vitro* whole cell binding assay with human AR transiently transfected in a monkey kidney cell line (COS).

DEGREE OF USE

Extensive use in reproductive medical field. Several publications now with fungicides and pesticides.

DURATION

A few days for entire assay, including cell culture preparation .

ASSAY STABILITY

Quite stable.

DOES IT METABOLIZE TOXICANTS

These cells can activate some antiandrogenic fungicides.

ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED

Yes, cell culture equipment and luminometer, robot optional.

ARE SPECIAL SKILLS/TRAINING NEEDED

Need to transfect cells.

HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY

Can be standardized fairly easily, especially with stable cell lines expressing hAR.

SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS

Sensitive, or more sensitive, than rat AR cytosolic binding assays.

ARE THERE KNOWN FALSE POSITIVES

Theoretically, chemical cytotoxicity may reduce radioligand binding to hAR, this could be interpreted as a false positive in the absence of appropriate controls.

OR FALSE NEGATIVES

Some toxicants requiring activation may not be detected.

SPECIFICITY-ARE THERE OTHER MECHANISMS TO PRODUCE THIS EFFECT (FALSE POSITIVES)

Cell death.

COMMENTS

Need controls in this and other similar assays for cell viability, and health. Must verify toxicant stability in media.

## **VI. Androgen and Anti-androgen - *In Vitro***

### **A. YAS-Yeast Androgen Screen**

#### **DESCRIPTION**

*In vitro* assay using yeast cells transformed with AR (whole or fragment), and a reporter (luc, J-gal, or etc.) construct.

#### **DEGREE OF USE**

Little use, one paper on use with toxicants. Results for sole xenoantiandrogen yielded a false negative.

#### **DURATION**

Short-term, 24 hours.

#### **ASSAY STABILITY**

Response likely varies greatly from subclone to subclone for certain types of xenoandrogens. Reservations same as for YES.

#### **DOES IT METABOLIZE TOXICANTS**

Unknown

#### **ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED**

Cell culture equipment and techniques.

#### **ARE SPECIAL SKILLS/TRAINING NEEDED**

Permanently transformed so may be easier than transient transfections, but *in vitro* training required.

#### **HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY**

Has not been standardized.

#### **SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS**

Insensitive to p,p' DDE.

#### **ARE THERE KNOWN FALSE POSITIVES OR FALSE NEGATIVE**

Detects antiandrogens as agonists, not as good as an AR-binding assay due to false negatives.

#### **SPECIFICITY-ARE THERE OTHER MECHANISMS TO PRODUCE THIS EFFECT (FALSE POSITIVES)**

Unknown

#### **COMMENTS**

As employed, is more like a binding assay than other transfected cell assays because it fails to discriminate between agonist and antagonists. Given limitations of transport (i.e., cellular mechanisms accounting for multidrug resistance), is a curious choice given the minimal information provided and high potential false negatives.

**B. CV-1 Cell Assay. A hAR Transcriptional Activation Assay in Mammalian Cell DESCRIPTION**

Transiently transfected assay measuring transcriptional activation using luciferase reporter and an hAR construct in a primate kidney cell line (CV-1).

**DEGREE OF USE**

Extensive use in reproductive medical field. Several publications now with fungicides and pesticides.

**DURATION**

A few days for entire assay, including cell culture preparation.

**ASSAY STABILITY**

Stable, with considerable experience.

**DOES IT METABOLIZE TOXICANTS**

These cells can activate some antiandrogenic fungicides.

**ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED**

Yes, freezer, incubator, and luminometer, robot optional.

**ARE SPECIAL SKILLS/TRAINING NEEDED**

Reported to be a difficult assay to initially establish. Need to transfect cells.

**HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY**

Can be standardized fairly easily, especially when stable cell lines have been established which will eliminate the need to transfect cells for each experiment.

**SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS**

Very sensitive.

**ARE THERE KNOWN FALSE POSITIVES**

Theoretically, cytotoxicity may induce apparent inhibition of DHT (this is true for many transfected cell reporter assays of antiandrogenicity,) hence, use constitutively active mutant receptor as a cytotoxicity control (decline in luc indicates cytotoxicity).

**OR FALSE NEGATIVES**

Some toxicants requiring activation may not be detected.

**SPECIFICITY-ARE THERE OTHER MECHANISMS TO PRODUCE THIS EFFECT (FALSE POSITIVES)**

Cell death

**COMMENTS**

Need controls in this and other similar assays for cell viability and health. Must verify toxicant stability in media.

**C. hAR Transactivation Assays Using Stable Cell Lines**

**DESCRIPTION**

Transcriptional activation assay using cells stably expressing a MMTV-luciferase reporter together with the hAR in a mammalian cell line such as a CV-1.

**DEGREE OF USE**

Under development, likely available in 6 months, could be automated for high throughput.

**DURATION**

24 hours

**ASSAY STABILITY**

Unknown to date, should be stable.

**DOES IT METABOLIZE TOXICANTS**

These cells can activate some antiandrogenic fungicides.

**ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED**

Yes, freezer, incubator, and luminometer, robot optional.

**ARE SPECIAL SKILLS/TRAINING NEEDED**

Do not need to transfect cells, but training required and cell techniques needed.

**HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY**

Not yet, but can be easily standardized.

**SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS**

Should be as sensitive, or more sensitive, than transient transfection assays because all the cells are responsive.

**ARE THERE KNOWN FALSE POSITIVES**

Theoretically, cytotoxicity may induce apparent inhibition of DHT-induced effects (this is true for many cell reporter assays of antiandrogenicity), hence, use constitutively active mutant receptor as a cytotoxicity control (decline in luc indicates cytotoxicity) .

**OR FALSE NEGATIVES**

Toxicants requiring activation may not be detected.

**SPECIFICITY-ARE THERE OTHER MECHANISMS TO PRODUCE THIS EFFECT (FALSE POSITIVES)**

Cell death.

**COMMENTS**

Need controls in this and other similar assays for cell viability and health. Must verify toxicant stability in media.

**D. Leydig Cell Culture**

**DESCRIPTION**

Determination of testosterone production in purified, isolated Leydig cells.

**DEGREE OF USE**

Limited to a several/few research laboratories.

**DURATION**

A few days

**ASSAY STABILITY**

Variable from lab to lab on degree of purification of Leydig cells and T production per cell.

**DOES IT METABOLIZE TOXICANTS**

No

**ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED**

Yes, quite a bit.

**ARE SPECIAL SKILLS/TRAINING NEEDED**

Requires skills that are not widely available.

**HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY**

No

SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS

Very sensitive to SIS, less sensitive, and to insensitive to estrogens.

ARE THERE KNOWN FALSE POSITIVES

Cell toxicants.

OR FALSE NEGATIVES

Toxicants that require metabolic activation.

SPECIFICITY-ARE THERE OTHER MECHANISMS TO PRODUCE THIS EFFECT (FALSE POSITIVES)

Cell membrane and second messenger effects.

**VII. Androgen and Anti-androgen - *In Vivo***

**A. Endocrine Challenge Test (Fail et al., 1995)**

DESCRIPTION

Repeated observation of serum T, LH and other hormones (with and without LH or GnRH challenge) in catheterized animal. Clearly the best way to detect altered hormone secretion *in vivo*.

DEGREE OF USE

Limited

DURATION

Few weeks.

ASSAY STABILITY

Stable, once the animal is catheterized.

DOES IT METABOLIZE TOXICANTS

Yes

ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED

Yes

ARE SPECIAL SKILLS/TRAINING NEEDED

Yes, quite difficult.

HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY

Has not been standardized.

SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS

More sensitive than single determination of serum hormones levels.

ARE THERE KNOWN FALSE POSITIVES

Stress reduces serum T, increases prolactin and corticosterone, effect that is rarely accounted for in most studies. Hormone levels are also subject to circadian effects.

COMMENTS.

Excellent, but specialized. Tail bleeds can also be used for repeated sampling from the same animal if only a small amount of serum is needed. This method also is not standardized and requires a great deal of practice.

**B. Super Apical Developmental Toxicity Test****DESCRIPTION**

Expose pregnant/lactating dam and examine hormone (AR, ER, SIS, Ah, and T3) sensitive endpoints in progeny up to puberty.

**DEGREE OF USE**

Such a protocol has been used at EPA, CIIT, NIEHS for xenoestrogens, environmental antiandrogens, Ah receptor agonists, phthalates and antithyroidal toxicants (PCBs and PTU).

**DURATION**

Relatively long (two to three months) as compared to other “screens,” would need to determine if it is quicker and cheaper to run this as opposed to several more focused, shorter-term assays.

**ASSAY STABILITY**

Unknown, but should be as good as any developmental test.

**DOES IT METABOLIZE TOXICANTS**

Yes

**ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED**

Yes, similar to needs for new multigen tests.

**ARE SPECIAL SKILLS/TRAINING NEEDED**

Multiplicity of *in vivo* techniques are required that are currently not used in toxicology labs, but they should be coming up to speed to implement new test guidelines.

**HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY**

Not standardized as used in different labs with respect to dosing or some of the assessments. Some standardization is now taking place between different labs.

**SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS**

Antiandrogenicity via AR, or SIS. In male progeny, AGD, areolas, nipples, reduced weight of prostate. Higher doses of AR-mediated, but not SIS, produce agenesis of prostate, undescended testis and agenesis of epididymis. Altered T production by testis, *ex vivo*.

Estrogenicity. Neonatal Uterine weight and gland development (?), vaginal opening, age at first estrus, vaginal cornification. Prostate size in male (?), and sperm production (at high dosage levels). SIS or Antiestrogenicity. Pregnancy loss, delay in deliver by dam, delay in VO and possible altered ovarian hormone production *ex vivo*.

Antithyroid. Reduced perinatal growth and brain size. Lower serum T4 and possibly T3, elevated TSH. Also detects functional developmental alterations induced by phthalates, TCDD and etc. The following endpoints are considered to be insensitive because they take too long to assess in a screen and/or have never been detected with a pesticide or toxic substance following developmental exposure (i.e., for ER-mediated: cancer, reduced AGD, hypospadias in male or female, undescended testes or any malformations).

**ARE THERE KNOWN FALSE POSITIVES.**

Very Apical Test used to screen for several mechanisms at once.

**OR FALSE NEGATIVES.**

Not likely, if designed properly.

**C. Pubertal Development in Male Rodent (Preputial Separation)****DESCRIPTION**

*In vivo* determination of age at puberty in male rat.

**DEGREE OF USE**

Data base includes drugs and antiandrogens and estrogenic pesticides and toxic substance (vinclozolin, pp DDE, methoxychlor, phthalates, TCDD, PCBs). Required endpoint in new multigenerational test guidelines.

**DURATION**

20-30 days

**ASSAY STABILITY**

Varies from block to block and from LE to SD by less than two days if designed carefully.

Delays of two to three days are generally significant with sample sizes of 10-12/group.

**DOES IT METABOLIZE TOXICANTS**

Yes

**ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED**

No

**ARE SPECIAL SKILLS/TRAINING NEEDED**

Some practice is required.

**HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY**

Has been, is almost as easy as puberty in female rat.

**SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS**

Slightly less sensitive than are some developmental AR-mediated alterations. Also moderately sensitive to xenoestrogens (for ER, PPS is less sensitive than growth, but much better than spermatogenesis and dosing duration is shorter).

**ARE THERE KNOWN FALSE POSITIVES**

Yes, this is an apical assay so anti-AR, ER, SIS inhibition, altered hypothalamic-pituitary function and Leydig cell toxicants will all delay this developmental landmark.

**OR FALSE NEGATIVES**

Unknown

**SPECIFICITY-ARE THERE OTHER MECHANISMS TO PRODUCE THIS EFFECT (FALSE POSITIVES)**

Nonspecific, apical test.

**COMMENTS**

Noninvasive test that could be coupled with both Hershberger test and biochemical assays.

**D. Hershberger Assay (1953)****DESCRIPTION**

*In vivo* measurement of effects of antiandrogenic/androgenic toxicants of androgen-dependent tissues in peripubertal/adult male rat. Weigh sex accessory glands and levator ani/bulbocavernosus muscle in T-implanted, castrate adult or intact juvenile male rat four to seven days after start of study. Can also measure serum T and DHT (to discriminate AR-mediated from SIS mechanisms and liver effects on metabolism), and biochemical indices (ODC) and tissue gene expression (TRPM2, C3).

## DEGREE OF USE

Extensive in drug development and recently in toxicology of antiandrogens.

## DURATION

One week.

## ASSAY STABILITY

Stable

## DOES IT METABOLIZE TOXICANTS

Yes

## ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED

Balance, scissors.

## ARE SPECIAL SKILLS/TRAINING NEEDED

Good necropsy and surgical skills.

## HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY

Has been standardized and validated.

## SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS

Responds as expected to moderate dosage levels of antiandrogenic pesticides.

## ARE THERE KNOWN FALSE POSITIVES

Yes, especially if conducted in intact peripubertal male. An apical test in intact animal. More specific in castrate but some endpoints are still affected by other mechanisms.

## OR FALSE NEGATIVES

None known.

## SPECIFICITY-ARE THERE OTHER MECHANISMS TO PRODUCE THIS EFFECT (FALSE POSITIVES)

Yes, prolactin, thyroid hormone, and estrogens affect some organ weights.

## COMMENTS

One of the best short-term assays for antiandrogens, along with pubertal development.

**VIII. Anti-androgen - Synthesis Metabolism****A. Testis/Ovary Culture *In Vitro* or *Ex Vivo***

## DESCRIPTION

Determination of testosterone production from testicular tissue from animals treated *in vivo* (*ex vivo*) or using *in vitro* dosing. Used for EDS, estrogens, antiandrogens, several other testicular toxicants, and substances that inhibit steroidogenesis. In female, minced ovary culture can be used *ex vivo* from pregnant (i.e., GD 14-16) or cycling females (proestrus for estradiol production).

## DEGREE OF USE

A few hundred publications over the last 25 years since the advent of RIAs for testosterone. Used in other vertebrates, as well as mammals. Used by several toxicology laboratories in addition to NHEERL-EPA.

## DURATION

One day for *in vitro*, longer for *ex vivo* (duration depends upon dosing).

**ASSAY STABILITY**

Depends on methodology.

**DOES IT METABOLIZE TOXICANTS**

Yes, for *ex vivo*, little or no metabolism for *in vitro*.

**ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED**

Incubator, freezer, necropsy equipment.

**ARE SPECIAL SKILLS/TRAINING NEEDED**

Some practice, but a rather simple assay that can be learned by competent technicians in a week or less. Need to be able to conduct RIAs for T, estradiol, and progesterone.

**HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY**

Has not been standardized between labs, but could be without too much difficulty.

**SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS**

Fairly sensitive.

**ARE THERE KNOWN FALSE POSITIVES**

Cell toxicants.

**OR FALSE NEGATIVES**

If metabolic activation is required then *in vitro* may not work.

**COMMENTS.**

These assays could be used rapidly to screen chemicals for ability to inhibit steroidogenesis.

**IX. Thyroid - Intrinsic Activity****A. TR Binding Assay****DESCRIPTION**

Determines whether a chemical can alter T3 binding to its nuclear receptor. Assay is performed on isolated nuclei. Nuclei can be isolated from liver of any species. In principal, these results would reveal competition for the binding site or an allosteric effect.

**DEGREE OF USE**

Extensive

**DURATION**

Four hours.

**ASSAY STABILITY**

Prepared nuclei are stable at -80°C for long periods. Assay is performed at RT.

**DOES IT METABOLIZE TOXICANTS**

No

**ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED**

Yes

**ARE SPECIAL SKILLS/TRAINING NEEDED**

Yes, but training period is not extensive.

**HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY**

Yes

**SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS**

Very sensitive; can be run over broad dose range.

**ARE THERE KNOWN FALSE POSITIVES**No, though chemicals that bind to TR *in vitro* may not affect TR signaling *in vivo*.**OR FALSE NEGATIVES**

Conditions of the assay must be standardized because a variety of factors (e.g., oxidation) blocks T3 binding to its receptor. In addition, solubility of lipophilic compounds may preclude binding in this *in vitro* system. Finally, chemicals that do not bind to the TR may still disrupt thyroid function or thyroid hormone action.

**SPECIFICITY**

High

**COMMENTS**

This assay should be practical to do with tissue (liver) from any vertebrate.

**Thyroid - *In Vitro*****X.****A. Whole Cell Binding Assays.**

Cell lines that express the thyroid hormone receptor can be used as a whole-cell binding assay. Generally,  $^{125}\text{I-T}_3$  is added to the media and after a short incubation period, nuclei are isolated and counted. The difference between a whole-cell assay and other binding assays is that the ligand must be taken up into the cell by stereospecific uptake sites that can be blocked by xenobiotics. Thus, this type of screen could potentially detect a broader array of chemicals that affect thyroid hormone action (i.e., those that bind to the TR and/or those that affect cellular uptake).

Cell lines from a number of vertebrates have been described to express the thyroid hormone receptor and, therefore, may be suitable. However, cell lines often exhibit a number of growth/maintenance characteristics that would make them more or less suitable for large screens, and this type of information would require further research to obtain.

**B. Stably Transfected Cell Lines.**

Cells that express the TR can also be stably transfected to provide a number of reporters that would respond to different kinds of thyroid responsive elements (TREs). An example would be GH<sub>3</sub> cells which have been used to study the role of thyroid hormone on the regulation of growth hormone gene expression. Because there are a number of TRE motifs, one goal would be to establish a cell line that would allow an easy screen of compounds that might affect the ability of TR to influence the expression of several types of regulatory elements. In principle, this type of assay could be established in cell lines from a variety of vertebrates with the same caveats listed above.

**C. Thyroid Hormone-Responsive Cells.**

Various cell lines change phenotype in response to thyroid hormone. PC12 cells are an example. This is a rat pheochromocytoma cell line which can be induced to become neuron-like in response to NGF. Thyroid hormone can block this change, but it requires transient transfection of the thyroid hormone receptor.

Another example is that of the XLT-15 cell line of Yaoita and Nakajima. These cells can be induced to undergo apoptosis by thyroid hormone. Thus, a screen may be developed to determine whether a compound can influence this induction.

**D. Specialized Cells.**

Various cell lines exhibit unique features that can be recruited for development of a screen. For example, FRTL-5 cells are derived from a rat thyrocarcinoma. Marinovich et al. [Marinovich 1995; 1153] have reported a clonal line that is stably transfected with the human thyroid peroxidase which can be inhibited by a number of chemicals (e.g., ethylenethiourea, a metabolite of dithiocarbamate pesticides).

**XI. Thyroid *In-vivo*****A. Short-Term Serum T<sub>4</sub>****DESCRIPTION**

Determines whether a chemical can alter circulating levels of thyroxine. Several mechanisms for this, including decreased half-life (displacing from serum carrier proteins, activating liver enzymes), or decreasing synthesis by effects on the thyroid itself. T<sub>4</sub> is more sensitive than T<sub>3</sub> or TSH. T<sub>3</sub> and TSH can be measured if T<sub>4</sub> is affected but there are a number of compounds that affect T<sub>4</sub> without affecting T<sub>3</sub> or TSH. Other indices would be required.

**DEGREE OF USE**

Extensive

**DURATION**

24 hours or more

**ASSAY STABILITY**

N/A

**DOES IT METABOLIZE TOXICANTS**

Yes

**ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED**

Yes, but T<sub>4</sub> kits are widely used clinically.

**ARE SPECIAL SKILLS/TRAINING NEEDED**

Yes, but training period is not extensive.

**HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY**

Yes

**SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS**

T<sub>4</sub> is not very sensitive in a short-term assay, though others may have more extensive knowledge on this.

ARE THERE KNOWN FALSE POSITIVES

No

OR FALSE NEGATIVES

Because the half-life of thyroid hormone in blood is long (~120h) and because some compounds may interfere with thyroid hormone synthesis (e.g., iodide uptake), short-term exposure may not be indicative of thyroid affects.

SPECIFICITY

High

COMMENTS

This could be coupled to a screen for reproductive effects.

**B. Long-Term Serum T<sub>4</sub>**

DESCRIPTION

Determines whether a chemical can alter circulating levels of thyroxine within a longer time-course. This could be performed on animals being evaluated for reproductive effects (see RTP proposal).

DEGREE OF USE

Extensive

DURATION

48 hours or more.

ASSAY STABILITY

N/A

DOES IT METABOLIZE TOXICANTS

Yes

ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED

Yes, but T<sub>4</sub> kits are widely used clinically.

ARE SPECIAL SKILLS/TRAINING NEEDED

Yes, but training period is not extensive.

HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY

Yes

SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS

Unclear

ARE THERE KNOWN FALSE POSITIVES

No

OR FALSE NEGATIVES

None that are known.

SPECIFICITY

Unknown

COMMENTS

This assay can be included in an experiment for reproductive effects.

**C. Thyroid Peroxidase****DESCRIPTION**

This description was contributed by Dan Sheehan (excerpted by TZ). The thyroid peroxidase has a broad substrate specificity, for all phenolic chemicals essentially. One group of prototypical ED's is the flavones and isoflavones; they inhibit thyroid peroxidase with IC<sub>50</sub>'s in the low micromolar range and can act as either competitive inhibitors or as suicide substrate inhibitors which inactivate the peroxidase. Either or both of these actions could lower T<sub>3</sub>/T<sub>4</sub> production, increase TSH and lead to goiter/carcinoma. A population of human infants consuming soy infant formula, which has a high isoflavone content, has been identified with Graves disease or Hashimotos thyroiditis, both autoimmune thyroid diseases diagnosed by goiter. The prevalence of soy formula consumption was twice as high in these patients as in controls who had consumed cows milk formula. The assay for inhibition is simpler, quicker, and cheaper than a receptor assay. It is a colorimetric assay of peroxidase activity in the presence of various concentrations of a chemical followed by graphical or computer solutions for IC<sub>50</sub>'s. It seems likely to me that other chemicals may act via the same mechanism. Also note that the phenolic "A" ring (or its equivalent) is crucial for estrogen receptor binding. Chemicals with a low ER RBA (?) (i.e., 1/1000 tha of E<sub>2</sub>) and a phenolic group would be active estrogens in the micromolar range AND would also possibly be active on the peroxidase. Given the co-occurrence of thyroid and reproductive problems in some geographical areas, this may be due to estrogenic activity of low affinity ER ligands which also have peroxidase inhibiting activity. While this assay does not account for all mechanisms leading to thyroid toxicity, just as an ER RBA assay does not, it is cheap, simple, and quick; and, I think, should be in the *in vitro* battery for the thyroid.

**DEGREE OF USE**

Extensive

**DURATION**

?

**ASSAY STABILITY**

N/A?

**DOES IT METABOLIZE TOXICANTS**

Yes

**ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED**

Modest

**ARE SPECIAL SKILLS/TRAINING NEEDED**

Yes, but training period is not extensive.

**HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY**

Yes

**SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS**

Unclear

**ARE THERE KNOWN FALSE POSITIVES**

No

**OR FALSE NEGATIVES**

Compounds may interfere with thyroid hormone action without affecting TPO.

**SPECIFICITY**

Unknown

**D. Malic Enzyme**

DESCRIPTION

DEGREE OF USE

Extensive

DURATION

?

ASSAY STABILITY

N/A?

DOES IT METABOLIZE TOXICANTS

Yes

ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED

Modest

ARE SPECIAL SKILLS/TRAINING NEEDED

Yes, but training period is not extensive.

HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY

Yes

SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS

Unclear

ARE THERE KNOWN FALSE POSITIVES

No

OR FALSE NEGATIVES

Compounds may interfere with thyroid hormone action without affecting TPO.

SPECIFICITY

Unknown

COMMENTS

See above

**E. Mammal Development**

DESCRIPTION

This assay can be performed on animals in which screens for reproductive effects are being evaluated as described in the RTP proposal. Simplest measures would be circulating T<sub>4</sub>, and brain weight.

DEGREE OF USE

Brain weight is not used as an index of thyroid hormone action during development, though brain weight is clearly affected by thyroid hormone action.

DURATION

?

ASSAY STABILITY

N/A?

DOES IT METABOLIZE TOXICANTS

Yes  
ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED  
Modest  
ARE SPECIAL SKILLS/TRAINING NEEDED  
Yes, but training period is not extensive.  
HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY  
Unclear  
SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS  
Unclear  
ARE THERE KNOWN FALSE POSITIVES  
Yes, compounds that affect nutritional status or eating. However, these may also influence thyroid hormone levels which are linked to nutritional status.  
OR FALSE NEGATIVES  
No?  
SPECIFICITY  
Unknown  
COMMENTS  
See above.

## **XII. Three Alternative Overviews**

*The following three overviews were submitted as alternatives to existing overviews and are included here as an addendum. (submitted by Dr. Soto)*

### **A. MCF7 Proliferation Assay (E-SCREEN)**

#### **DESCRIPTION**

Measures proliferation of cells in culture in response to estrogens.

#### **DEGREE OF USE**

Used in various laboratories in the U.S.A., Europe and Japan. Many chemicals studied; several toxicants were discovered to have estrogenic properties using this method. Extensive published data on toxicants with estrogenic activity.

#### **DURATION**

From seeding the cells to harvesting cells=5 to 7 days. It requires 4 manipulations only: (1) seeding, (2) changing to test medium 24 hours later, (3) single-step harvesting (or staining in situ) and (4) counting (or reading in an ELISA plate reader).

#### **ASSAY STABILITY**

Very stable in the labs that started with a line/subline of proven sensitivity. Clones reported to have stable activity for over ten years.

#### **DOES IT METABOLIZE TOXICANTS?**

Not thoroughly characterized. However, some proestrogens (methoxychlor, bisphenol-A dimethacrylate, alkylphenol-monoethoxylates, non-hydroxylated PCBs) were reported to have activity in this assay.

**ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED?**

Like all assays performed with vertebrate cell cultures, it requires laminar flow hood, CO<sub>2</sub> incubator, cell freezing storage, inverted microscope, cell counting device, and a detector for the specific end point measured (it may use an electronic cell counter or an ELISA plate reader if using sulforhodamine-B assay or other colorimetric assay).

**ARE SPECIAL SKILLS/TRAINING NEEDED?**

Like all assays using cell culture, it requires the ability of maintaining the culture stocks, periodic freezing after several passages to maintain the cultures in case of infections or other mishaps. The assay itself is very easy to perform.

**HAS IT BEEN STANDARDIZED EASILY?**

It is highly reproducible from assay to assay. It is used in several labs in the USA and Europe. By using responsive cells to begin with, it has provided comparable results when a battery of 20 coded chemicals were tested by several labs (manuscript in preparation; Project Coordinator: Philip Grandjean, Odense University, Denmark). Competent labs appear to get similar results. Cell of appropriate phenotype can be easily distributed.

**SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS**

Very sensitive; one of the most sensitive assays (E2 EC<sub>50</sub>=10-15 pM range; maximal activity 100 pM).

**ARE THERE KNOWN FALSE POSITIVES?**

In competent labs no false positives were found among growth factors. Testosterone is the only steroid that shows activity at TM concentrations. When assaying for antagonists, endocrinologists have used a two-step method to assess whether or not the effect is truly antiestrogenic regardless of whether the end point is inhibition of estrogen-induced cell proliferation or inhibition of estrogen induction of a gene product: 1) assess the effect of a range of doses of the test compound together with the minimal estrogen dose needed for maximal induction of the gene product (inhibition), and 2) assess the effect of increasing doses of estradiol administered together with the dose of toxicant found to induce maximal inhibition (estradiol rescue).

**OR FALSE NEGATIVES?**

False negatives due to toxicity were not found using GLP (it only takes looking at the cells with the inverted microscope to detect unspecific toxicity). TCDD does have toxic effects both in the presence and absence of estrogens. Various substances that require metabolic activation like alkylphenols polyethoxylates may be missed.

**SPECIFICITY-ARE THERE OTHER MECHANISMS OF ACTION TO PRODUCE THIS EFFECT (FALSE POSITIVES)?**

So far, all substances found to be estrogenic with the E-SCREEN assay that were also tested using gene activation assays were found to be consistently estrogenic.

**COMMENTS**

As with all bioassays, it requires obvious good laboratory practices. In the case of rodent bioassays it is important to work with healthy animals, appropriate light cycles, using feeds that do not contain estrogens, etc. When working with cells in culture, one has to start with a subline that expresses the appropriate phenotype. To maintain the phenotype, serum used for

cell propagation has to be checked before using it for propagation (therefore, laboratories stock “good serum” to last for one year). Similarly, stocks have to be frozen periodically, and the charcoal-dextran stripped serum (which can be stored frozen in aliquots for up to one year, should be checked once before use). Recently, it was shown that recombinant serum albumin may be used instead of charcoal-dextran stripped serum.

The assay can be automated, and different labs use 12-, 24- or 96-well plates, depending on whether one uses cell numbers, sulforhodamine-B staining or MTT reaction as the end point). Also, cells can be fixed in situ at the end of the experiment and stained days or weeks later.

## **B. MVLN Assay. Stably Transfected Reporter Gene Assay in Mammalian Cells**

*(submitted by Dr. Soto)*

### **DESCRIPTION**

The assay utilizes a mammalian cell line (MCF7 with endogenous ER) that has been stably transfected with an ER-specific reporter gene (Vit-luc).

### **DEGREE OF USE**

Used in various laboratories for pharmaceutical and environmental research. No publications are listed in Medline as yet attesting its use for estrogenic toxicants.

### **DURATION**

From seeding the cells to harvesting lysate and Luc activity takes two to three days. The following manipulations are required: (1) seeding and exposure to charcoal-dextran stripped serum containing tamoxifen for 24-48 hours; (2) changing the medium to tamoxifen-free for 12 hours (or, treat for 1-2 weeks with charcoal-dextran stripped serum,); (3) incubate with test substance for 24 or more hours; (4) finally harvest cells; (5) homogenize them; (6) read assay; and (7) run a protein assay.

### **ASSAY STABILITY**

Like the parent cell line MCF7, it should be stable when good laboratory practices are followed. It has been stable for 30 passages. Exposure to tamoxifen renders these cells unable to express estrogen induction of the reported gene.

### **DOES IT METABOLIZE TOXICANTS?**

Not thoroughly characterized. It may maintain same capabilities as those of the parental cell line.

### **ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED?**

Like all assays performed with vertebrate cell cultures, it requires laminar flow hood, CO<sub>2</sub> incubator, cell freezing storage, inverted microscope, cell counting devise, and a detector for the specific end point measured (luminometer to measure Luc activity).

### **ARE SPECIAL SKILLS/TRAINING NEEDED?**

Like all assays using cell culture, it requires the ability of maintaining the culture stocks, periodic freezing after several passages to maintain the cultures in case of infections or other mishaps. The assay itself is easy to perform.

### **HAS IT BEEN STANDARDIZED EASILY?**

No published results are available on the performance of this assay to detect estrogenic toxicants. Stably transfected cells can be easily distributed.

### **SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS**

Very sensitive; EC50 for E2 in the 20 pM range; maximal activity of E2 was reported at 1 nM.  
ARE THERE KNOWN FALSE POSITIVES?

Hypothetically, ER may be activated by non-estrogenic agents through phosphorylation pathways. When assaying for antagonists, endocrinologists have used a two-step method to assess whether or not the effect is truly antiestrogenic regardless of whether the end point is cell proliferation or inhibition of estrogen induction of a gene product: (1) assess the effect of a range of doses of the test compound together with the minimal estrogen dose needed for maximal induction of the gene product (inhibition); and (2) assess the effect of increasing doses of estradiol administered together with the dose of toxicant found to induce maximal inhibition (estradiol rescue).

OR FALSE NEGATIVES?

Similarly to the parental cell line, false negatives due to toxicity should be excluded by GLP (it only takes looking at the cells with the inverted microscope to detect unspecific toxicity). It is suspected that like in the parent cell line TCDD may have toxic effects both in the presence and absence of estrogens. Various substances that require metabolic activation like alkylphenols polyethoxylates may be missed.

SPECIFICITY-ARE THERE OTHER MECHANISMS OF ACTION TO PRODUCE THIS EFFECT (FALSE POSITIVES)?

Hypothetically, ER may be activated by non-estrogenic agents through phosphorylation pathways.

COMMENTS

Specific ER activation. As with all bioassays, it requires obvious good laboratory practices. In the case of rodent bioassays it is important to work with healthy animals, appropriate light cycles, using feeds that do not contain estrogens, etc. When working with cells in culture, one has to start with a subline that expresses the appropriate phenotype. To maintain the phenotype, serum used for cell propagation has to be checked before use (therefore, laboratories stock "good serum" to last for at least one year). Similarly, cell stocks have to be frozen periodically.

The assay can be automated; can be done in 12-, 24- or 96-well plates.

**C. MCF7-AR1 Assay. MCF7 Cells Stably Transfected With "Wild Type" Androgen Receptor.** (submitted by Dr. Soto)

DESCRIPTION

The assay utilizes a mammalian cell line (MCF7) stably transfected AR. These cells proliferate maximally in serumless medium supplemented with insulin and transferrin. Androgens inhibit their proliferation; antiandrogens abolish the inhibitory effect of androgens.

DEGREE OF USE

This method was just published.

DURATION

From seeding cells to harvesting them, it takes 5 days.

ASSAY STABILITY

Like the parent cell line MCF7, it should be stable when good laboratory practices are followed. It has been stable for 4 years.

**DOES IT METABOLIZE TOXICANTS?**

Not thoroughly characterized. It should keep the same capabilities as those of the parental cell line.

**ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED?**

Like all assays performed with vertebrate cell cultures, it requires a laminar flow hood, CO<sub>2</sub> incubator, cell freezing storage, inverted microscope, cell counting devise, and a detector for the specific end point measured (it may use an electronic cell counter or an ELISA plate reader when using sulforhodamine-B assay or other colorimetric assay).

**ARE SPECIAL SKILLS/TRAINING NEEDED?**

Like all assays using cell culture, it requires the ability of maintaining culture stocks, periodic freezing after several passages to maintain the cultures in case of infections or other mishaps. The assay itself is easy to perform.

**HAS IT BEEN STANDARDIZED EASILY?**

It can be standardized easily, since cells express AR constitutively. No published results are available on the performance of this assay to detect toxicants that are androgen agonists or antagonists. Stably transfected cells can be easily distributed.

**SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS**

Very sensitive; EC50 for DHT in the pM range.

**ARE THERE KNOWN FALSE POSITIVES?**

In theory, hyperphysiological doses of glucocorticoids may bind to the AR and this may activate AREs.

**OR FALSE NEGATIVES?**

As with the parental cell line, false negatives due to toxicity should be excluded by GLP (it only takes looking at the cells with the inverted microscope to detect unspecific toxicity). In addition, since androgens inhibit cell proliferation, “rescue” from inhibition by an antiandrogen may help differentiating toxicity from androgenicity. Substances that require metabolic activation may be missed.

**SPECIFICITY-ARE THERE OTHER MECHANISMS OF ACTION TO PRODUCE THIS EFFECT (FALSE POSITIVES)?**

Probably hyperphysiological doses of glucocorticoids.

**COMMENTS**

Like all bioassays, it require good laboratory practices. In the case of rodent bioassays it is important to work with healthy animals, appropriate light cycles, using feeds that do not contain estrogens, etc. When working with cells in culture, the cell line should express the appropriate phenotype. To maintain the phenotype, serum used for cell propagation has to be checked before use (therefore, laboratories stock “good serum” lots for at least one year).

Similarly, cell stocks have to be frozen periodically.

The assay can be automated; can be done in 12-, 24- or 96-well plates.

**XIII. Overview of Non-Mammalian Screens**

**A. Procedures Using Birds and Reptiles to Determine Endocrine Disruptive Action**

The number of procedures available are limited for taxa other than mammals. In part, this is because many procedures used in mammals have just not been tried in other taxa, as well as because they are not appropriate for egg-producing vertebrates or any phyla other than Vertebrata. Hence, the procedures below have been divided into two matrices with identical structure as the mammalian matrix, but including both birds and reptiles as subjects. The first matrix contains procedures that have already been used to determine endocrine action of contaminants, and the second contains procedures that could be used, but have not. The procedures in the second matrix need varying amounts of development to be ready for inclusion in a screening and testing program. The amount of development needed is estimated in the “comments” section.

Also, the number and variety of *in vitro* procedures that have been applied to wildlife is orders of magnitude smaller than used with mammalian material. This may be a particular problem for the design of a comprehensive set of **screening** procedures, because the screening battery is intended to cull out from a list of chemicals to be tested, all those chemical that have no intrinsic endocrine activity. Hence, the screening battery should be comprehensive for all types of endocrine action (or as many as possible). Thus the availability of short duration procedures using non-mammalian material that could be used as screens would seem crucial as the screening and testing program is intended to be protective of all taxa, not just humans and other mammals. The issue of the homology of steroid hormone and receptor structure across vertebrates classes needs to be explored more fully to help design an adequately comprehensive program, especially for the screening phase. At this date, there is no real choice to be made as there are very few if any procedures using non-mammalian material that could be used as a screening tool. This area needs thoughtful development. In addition to development of non-mammalian vertebrate *in vitro* procedures, invertebrates in whole animal “biological activity” tests could be exploited, which by dint of the very short life cycle, of *Daphnia* for instance, could be economically used in a screening battery.

**B. Overview of Endocrine Disruptor Relevant Screens to the Lower Vertebrates and Invertebrates**

Unlike the many and wide array of screens available for mammals, few exist for the animals outside that taxonomic group. Some assays do exist which can be employed with differing degrees of specificity and sensitivity. Predominantly, the assays available for these animals will be *in vivo* and somewhat apical. Although this means these assays will not provide detailed mode of action information, the endpoints will be relevant for interpreting “adverse” effects.

For the non-mammalian vertebrates, vitellogenin assays are available and will generally provide suggestive evidence of estrogen-related disruptions. However, there is some evidence of thyroid hormone involvement with vitellogenin production which may compromise any conclusive evidence of an estrogen specific action. Nonetheless, compounds which affect a change in normal

vitellogenin levels should be captured in a screening program for more definitive investigation of the effect and its relevance.

Existing standardized tests for evaluating conventional toxicities are also included here because of the availability of such information for certain compounds (e.g., pesticides). Information from these tests can be used to screen for suggestive endocrine mediated effects to be flagged for further investigation.

## **Amphibian Screens**

### **XIV.**

#### **A. Vitellogenin Assay**

##### **DESCRIPTION**

An assay which measures the amount of an egg yolk protein precursor in males as an indicator of estrogenic activity.

##### **REFERENCES**

Selcer, "Vitellogenin induction in frogs by immersion in xenobiotic estrogens," *Am. Zool.*, 36, 1996, p. 5. Peterson, G.L., *Determination of total protein: Methods of Enzymology*, 91, 1993, pp. 95-121.

##### **DURATION**

> 72 hours

##### **EQUIPMENT**

Wet lab, antibody, immunology lab

##### **STAFF SKILLS**

Can be trained

##### **COST**

Modest

##### **AVAILABILITY**

Limited by specificity of antibody, but Heppell et al. efforts at developing a "universal" antibody appear promising

##### **SENSITIVITY**

Good

##### **SPECIFICITY**

Good for estrogen activity, but thyroid hormones may also be involved

##### **STANDARDIZATION**

Not yet, but could be made without undue difficulties

##### **RELATEDNESS**

Primarily estrogen box, more research is needed to ascertain whether thyroid or androgen activity is or is not connected

#### **B. Frog Embryo Teratogenesis Assay Xenopus (FETAX)**

##### **DESCRIPTION**

A 96-hour whole embryo teratogenesis screening assay. Because the exposure is through primary organogenesis all developmentally important processes are taken into account.

## REFERENCES

Dumont et al., "Frog Embryo Teratogenesis Assay - Xenopus (FETAX)," *Shortterm Bioassays in the Analysis of Complex Environmental Mixtures III*, 1983. Plenum, ASTM, *Standard guide for conducting the Frog Embryo Teratogenesis Assay - Xenopus (FETAX)*, E, 1991, pp. 1439-91.

## DURATION

96 hours

## EQUIPMENT

General wet lab and microscopy lab

## STAFF SKILLS

Specialized knowledge of amphibian embryology and histology

## COST

Modest

## AVAILABILITY

Fair

## SENSITIVITY

Fair, not fully comprehensive for all estrogenic, androgenic, or thyroid related effects

## SPECIFICITY

Does not distinguish hormonal from non-hormonal developmental effects

## STANDARDIZATION

Yes through ASTM

## RELATEDNESS

Apical for some estrogenic, androgenic, and thyroid related effects other

**C. Amphibian Metamorphosis Assay (Conceptual)**

## COMMENTS

Similar to a FETAX like assay but focused on tadpole metamorphosis, perhaps specifically on tail resorption. Apical for thyroid related effects.

**D. Frog *In Vivo* Screening Assay (Conceptual)**

## COMMENTS

Similar in concept to what is proposed for fishes evaluating endpoints such as gonadosomatic index, secondary sex characteristics, oocyte maturation, plasma steroids, and plasma vitellogenin. Apical for estrogen, androgen, and thyroid related effects.

**E. Metamorphosis**

## DESCRIPTION

Determines whether a chemical can affect the thyroid hormone-dependent process of metamorphosis.

## DEGREE OF USE

Extensive

**DURATION**

Depends on species available. *Xenopus* or *Rana* would require about a week of treatment. *Scaphiopus* would require less time (24 hours), but may not be widely available.

**ASSAY STABILITY**

N/A?

**DOES IT METABOLIZE TOXICANTS**

Yes

**ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED**

Modest

**ARE SPECIAL SKILLS/TRAINING NEEDED**

Yes, but training period is not extensive

**HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY**

Yes

**SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS**

yes

**ARE THERE KNOWN FALSE POSITIVES**

No

**OR FALSE NEGATIVES**

Unclear

**SPECIFICITY**

Unknown

**COMMENTS**

The ease of compound administration may make this attractive. The use of  $\pm$  T3/T4 paradigm would allow for identification of thyroid action disruption. Endpoints (e.g., hind-limb growth rate) are  $\int$ egrated measures; thus, the screen would detect compounds that affect thyroid hormone action along the entire pathway. There are clearly a number of endpoint which may be more or less sensitive or reliable. Structure of internal organs (liver, pronephros), and production of urea are two.

**Bird Screens****XV.****A. Avian Egg-Injection Assay****TEST AND FUNCTION:**

The effects of steroids (and EDCs) on development of the reproductive tract and steroid concentrations. Subject are various species of wildlife. Exposure is by egg-injection before organogenesis; responses are measured at hatching and include the morphology histology of the reproductive tract of males (females may be useful also), and plasma steroid concentrations. It can be extended into the breeding age of the affected animal to look check for functional impairment.

**REFERENCES:**

Fry, D.M., C.K. Toone, S.M. Speich, and R.J. Peard, "Sex ratio skew and breeding patterns of gulls: demographic and toxicological considerations," *Studies in Avian Biology* 10, 1987, pp. 26-43.

Nisbet et al., 1996.

Several other studies now in progress using similar methodology with other species.

**DURATION:**

Depends on the length of incubation (~ three weeks minimum), and availability of eggs: wild birds usually not easily available unless a breeding colony exists.

**EQUIPMENT:**

Incubator, hatchabator, general lab facilities and facilities for RIAs.

**STAFF SKILLS:**

Animal husbandry skills, general dissection and microscope skills, tissue handling and preparation for sectioning, biochemical expertise for RIA work.

**AVAILABILITY:**

Wide.

**SENSITIVITY:**

Good for estrogens.

**SPECIFICITY:**

Estrogenic effects in males: possibly non-aromatizable androgen effects in females.

**STANDARDIZATION:**

Needs work, especially in preparation and analysis of histological responses.

**OTHER:**

The choice of subject species is crucial for operational ease, practicality and cost control. Embryos must be large enough at hatching to get a gonad that is easily handled and that has enough blood to collect for RIA on the plasma. Very similar responses to procedure R2. Compare to A2.

**B. Japanese Quail Early Life Stage**

**TEST AND FUNCTION:**

The effects of steroids (and EDCs) on development of sexual maturity in Japanese quail, and, if desired, of the reproductive tract and plasma steroid concentrations in siblings.

This tests the time to onset of maturity as measured by secondary sex characters (cloacal or foam gland size) and behavior (crowing) in males; all are testosterone dependent.

Exposure varies, see below.

**REFERENCES:**

Ottinger, M.A. and H.J. Brinkley, "Testosterone and sex related behavior and morphology: Relationship during maturation in the adult Japanese quail," *Hormones and Behavior* 11, 1978, pp. 175-182; and other references.

**DURATION:**

~18 days incubation + 60 days to maturity.

**EQUIPMENT:**

Avian husbandry, general lab, tape recorder.

**STAFF SKILLS:**

Husbandry, general lab.

**AVAILABILITY:**

Quail are widely available; strains can be different, recommend out bred line.

**SPECIFICITY:**

Depending on the timing of exposure it can be made specific to estrogen OR androgens:

- if chicks are implanted or injected with EDC, male time to maturity is androgen sensitive;
- if eggs are injected, the gonadal development of male offspring is impaired by estrogens (and possibly non-aromatizable androgens in female offspring, although this has not been demonstrated [ matrix 2]); this is similar to procedure A1. Estrogenic effects on time to maturity in males is not known.

**STANDARDIZATION:**

This test has been used widely and can be easily standardized further .

**OTHER:**

Excellent background information on the endocrinology of Japanese quail; this procedure might be developed into an Androgen *antagonist* assay in males with co-administration of EDC and androgen, and androgen alone as control [matrix 2].

**C. Japanese Quail Androgenicity Screen****TEST AND FUNCTION:**

Foam gland size and crowing behavior in photo-regressed adult males to look at the Androgenic effect of EDCs on peripheral targets, using Japanese quail. This procedure uses individuals that are not secreting GnRH, and thus the HPG axis is shut down.

**REFERENCES:**

[Matrix 2]

**DURATION:**

Two weeks to regress; expose for two days, responses in five to seven days.

**EQUIPMENT:**

Animal husbandry, photoperiod control, general lab.

**SPECIFICITY:**

Measures androgenic effects only.

**STANDARDIZATION:**

Good potential.

**RELATEDNESS:**

Could be made into an Androgen *antagonist* procedure, with the co-administration of testosterone + EDC, and using testosterone treatment alone as a control; may not be very sensitive, and there is likely to be a threshold effect of the administered testosterone, making the test for an antagonist less sensitive.

**OTHER:**

Almost ready to go; needs testing with EDCs.

**D. Vitellogenin Production in Female Japanese Quail**

TEST AND FUNCTION:

Vitellogenin production in photo-regressed adult females to look at the estrogenic effect of EDCs on peripheral targets, using Japanese quail.

REFERENCES:

[Matrix 2]

DURATION:

Two weeks to regress; expose for two days, vitellogenin response in 24 hours.

EQUIPMENT:

Animal husbandry, photoperiod control, electrophoresis lab.

STAFF SKILLS:

As above.

SPECIFICITY:

Specific for estrogen binding peripherally; could be made into a test for the E-antagonist with co-administration of E2 and the potential EDC, along with E2 to the controls.

STANDARDIZATION:

Good potential: husbandry can be made standard, but a common source of antibody is needed; ring testing probably needed for electrophoresis

OTHER:

Needs more development than A3. Could be used as a procedure for estrogen antagonists with co-administration of estrogen and using estrogen alone as a control.

**E. Vitellogenin Production in Adult Male Birds: Japanese Quail, Chickens.**

REFERENCES:

Robinson, G.A. and A.M.V. Gibbins, "Induction of vitellogenesis in Japanese quail as a sensitive indicator of the estrogen mimetic effect of a variety of environmental contaminants," *Poultry Science*, 63, 1984, pp. 1529-1536. (and other references )

Heppell, S.A., M.D. Denslow, L.C. Folmar and C.V. Sullivan, "Universal assay of vitellogenin as a biomarker of environmental estrogens," *Environ. Health Perspectives*, 103 (Suppl 7), 1996, pp. 9-15.

[see also: Matter, J.M., A.B. Anthony, K. Alonso, and R.L. Dickerson, "TCDD suppression of vitellogenin synthesis in female hens," Abstract. 17th Annual meeting of SETAC, 1996.]

DURATION:

Eight to ten days.

EQUIPMENT:

Animal facilities, electrophoresis equipment.

STAFF SKILLS:

Animal husbandry, electrophoresis, general lab.

AVAILABILITY:

Depends on antibody: birds widely available.

SENSITIVITY:

"High", but o,p-DDT did not induce vitellogenesis.

**SPECIFICITY:**

High.

**OTHER:**

Looks pretty good; a little shorter duration than A5 (photo-regressed females) but relative sensitivity is unknown. Lots of current work on different species of wildlife that is not published

**F. Avian “Plaque Assay”**

**TEST AND FUNCTION:**

Avian “Plaque assay”; slices of brain are prepared *in vitro* and infused with steroids, peptides or EDCs and GNRH production is measured. Exploits the negative feedback control of steroids on GNRH production.

**REFERENCES:**

Personal communication; Tom Porter’s lab at Texas A&M; reprints in the mail for more details.

**AVAILABILITY:**

Only this lab uses the technique, as far as can be told.

**OTHER:**

This assay should be explored as it is one of the only *in vitro* assays for birds. Drawbacks include killing birds for brains, independence of different slices from the same brain is questionable, and the expertise rather local, I believe. Needs development.

**G. Avian Cell Culture**

**TEST AND FUNCTION:**

To test the action of steroids and EDCs on the handful of immortalized avian cell lines. In general, steroidogenic endpoints have not been identified, much less evaluated for sensitivity. Further only one avian cell line (fibroblasts) is spontaneously immortal and not chemically or virally transformed, hence is probably the best for results relevant to an intact animal. Again, steroidogenic responses have not been identified in these fibroblasts (are any expected in a fibroblast?).

**REFERENCES:**

Personal communication, Doug Foster, U Minn.

**AVAILABILITY:**

Patented, but is willing to license for this use.

**OTHER:**

Needs development, but promising.

**H. Chicken Early Life Stage**

**TEST AND FUNCTION:**

Coxcomb size, vent sex and gonadal morphology and histology of chickens; EDCs or steroid administered by injecting the eggs before organogenesis; similar to B1 and B2.

**REFERENCES:**

Elbrecht, A., R.G. and Smith, "Aromatase enzyme activity and sex determination in chickens," *Science*, 225, 1992, pp. 467-470.

Snedecor, J.G., "A study of some effects of sex hormones on the embryonic reproductive system and comb of the white leghorn chick," *J. Exptl. Zool.*, 110, 1949, pp. 205-246.

- and others..

**DURATION:**

Long: 23 weeks to maturity, but for morphology of the reproductive tract, can collect material from hatchlings after 21 day incubation.

**EQUIPMENT:**

Animal facilities, RIA, tissue handling.

**STAFF SKILLS:**

Husbandry, RIA techniques, histological imbedding and slicing.

**AVAILABILITY:**

No problem.

**SPECIFICITY:**

For Estrogenic activity in males chicks, and for aromatase inhibition in females; aromatizable androgen has no effect (but DHT, and hence other non- aromatizable androgens ?). Needs development for possible A+ procedure.

**STANDARDIZATION:**

Easy for coxcomb, subjective assessments of gonadal morphology can be made more quantitative.

**OTHER:**

Coxcomb assay is a classic and has been widely used in the past, but it is very time consuming as maturity is reached not until five months; however, differences can be seen in the incompletely mature cock and may be useful. A nice twist exploited by Elbrecht and Smith is to use a cross that produced sexual dimorphism in feather color which was independent of steroid dependent characters, thus revealing genetic sex. In some studies the EDC has been topically applied to the comb directly. Needs development for possible A+ procedure.

**I. Cartilage Growth in Chick**

**DESCRIPTION**

Determines whether a chemical can alter the response of chick cartilage to thyroid hormone.

**DEGREE OF USE**

Extensive.

**ASSAY STABILITY**

N/A?

**DOES IT METABOLIZE TOXICANTS**

Yes.

**ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED**

Modest.

**ARE SPECIAL SKILLS/TRAINING NEEDED**

Yes, but training period is not extensive.  
HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY  
Yes.  
SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS  
Unclear.  
ARE THERE KNOWN FALSE POSITIVES  
No.  
OR FALSE NEGATIVES  
Compounds may interfere with thyroid hormone action without affecting cartilage.  
SPECIFICITY  
Unknown.

## **Fish Screens**

### **XVI.**

#### **A. Vitellogenin Assay**

##### **DESCRIPTION**

An assay which measures the amount of an egg yolk protein precursor in males as an indicator of estrogenic activity.

##### **REFERENCES**

Sumpter, J. P., "The purification, radioimmunoassay and plasma levels of vitellogenin from the rainbow trout. *Salmo gairdneri*," *Proceedings of the Ninth International Symposium on Comparative Endocrinology*, Hong Kong University Press, Hong Kong, 1985, pp. 335-357.

Peterson, G. L., *Determination of total protein: Methods of Enzymology*, 99, 1993, pp. 95-121.

##### **DURATION**

> 72 hours.

##### **EQUIPMENT**

Wet lab, antibody, immunology lab.

##### **STAFF SKILLS**

Can be trained.

##### **COST**

Modest.

##### **AVAILABILITY**

limited by specificity of antibody although Heppell *et al.* are developing a "universal" antibody.

##### **SENSITIVITY**

Good.

##### **SPECIFICITY**

Good for estrogen activity, but thyroid hormones may also be involved.

##### **STANDARDIZATION**

Not yet, but could be made without undue difficulties.

RELATEDNESS

Primarily estrogen box, more research is needed to ascertain whether thyroid or androgen activity is or is not connected.

**B. In Vitro Vitellogenin Assay**

DESCRIPTION

A procedure isolating trout hepatocytes, treating them with a xenobiotic, and then measuring the amount of vitellogenin secreted into the culture medium.

REFERENCES

Pelissero et al., "Vitellogenin synthesis in cultured hepatocytes: an *in vitro* test for the estrogenic potency of chemicals," *J. Steroid Biochemistry and Molecular Biology* 44, 1993, pp. 263-272.

EQUIPMENT

Immunology lab.

STAFF SKILLS

Moderate, can be trained.

AVAILABILITY

Fair.

SENSITIVITY

Good.

SPECIFICITY

Good for estrogen activity.

STANDARDIZATION

Practical.

RELATEDNESS

Applicable to estrogen boxes .

**C. In Vivo Screening Assay**

Refer to Peter Thomas' write up.

**D. Early Life Stage Test**

DESCRIPTION

Newly fertilized eggs are exposed to a test chemical through hatching and early development and growth of the juvenile fish. Endpoints measured are hatching success, survival, and growth.

REFERENCES

OECD 210, EPA 850.1400, ASTM E 1241-92.

DURATION

31 - 72 days.

EQUIPMENT

Wet lab sufficient for flow-through studies.

STAFF SKILLS

General.

**AVAILABILITY**

Commercially available.

**SENSITIVITY**

Good.

**SPECIFICITY**

Poor.

**STANDARDIZATION**

Yes.

**RELATEDNESS**

Apical for all boxes, but does not differentiate hormonal from non-hormonal driven effects and is not fully comprehensive.

**E. Embryo and Sac Fry Test**

**DESCRIPTION**

This is a short-term test in which the life stages from the newly fertilized egg to the end of the sac-fry stage are exposed. OECD 212.

**DURATION**

8 to 55 days.

**EQUIPMENT**

Wet lab.

**STAFF SKILLS**

General.

**AVAILABILITY**

Commercially available.

**SENSITIVITY**

Unknown.

**SPECIFICITY**

None.

**STANDARDIZATION**

Yes.

**RELATEDNESS**

Apical for general fish health.

**F. Partial Life Cycle Test**

**REFERENCES**

Standard Methods for the Examination of Water and Wastewater, APHA, AWWA, and WPCF 810B, 1985, p. 2.

**DURATION**

Long term, >250 days.

**EQUIPMENT**

Wet lab.

**STAFF SKILLS**

Experienced.

**COST**

High.

**AVAILABILITY**

Commercially available.

**SENSITIVITY**

Good.

**SPECIFICITY**

Apical, but does not address transgenerational effects.

**STANDARDIZATION**

Fair.

**RELATEDNESS**

Applicable to all boxes, but more appropriate in definitive testing as a screen it should only be used if the test has already been completed.

**G. Full Life Cycle Test**

**REFERENCE**

Standard Methods for the Examination of Water and Wastewater, APHA, AWWA, and WPCF, 810B, 1985, p. 3.

**DURATION**

Long term, >250 days.

**EQUIPMENT**

Wet lab.

**STAFF SKILLS**

Experienced.

**COST**

High.

**AVAILABILITY**

Commercially available.

**SENSITIVITY**

Good.

**SPECIFICITY**

Apical, but does not address transgenerational effects.

**STANDARDIZATION**

Fair.

**RELATEDNESS**

Applicable to all boxes, but more appropriate in definitive testing as a screen it should only be used if the test has already been completed.

**H. Flounder Metamorphosis**

**DESCRIPTION**

Determines whether a chemical can affect the thyroid hormone-dependent process of metamorphosis.

**DEGREE OF USE**

Extensive.  
DURATION  
Seven days.  
ASSAY STABILITY  
N/A?  
DOES IT METABOLIZE TOXICANTS  
Yes.  
ARE SPECIAL EQUIPMENT/REQUIREMENTS MANDATED  
Modest.  
ARE SPECIAL SKILLS/TRAINING NEEDED  
Yes, but training period is not extensive.  
HAS IT BEEN OR CAN IT BE STANDARDIZED EASILY  
Yes.  
SENSITIVITY TO LOW DOSES OR WEAKLY ACTIVE CHEMICALS  
Yes.  
ARE THERE KNOWN FALSE POSITIVES  
No.  
OR FALSE NEGATIVES  
Unclear.  
SPECIFICITY  
Unknown.  
COMMENTS

The ease of compound administration may make this attractive. The use of  $\pm$  T3/T4 paradigm would allow for identification of thyroid action disruption. Endpoints (e.g., eye migration) are  $\int$ egrated measures; thus, the screen would detect compounds that affect thyroid hormone action along the entire pathway.

**I. In Vitro Steroid Receptor Competition Assay**

No information submitted.

**J. In Vitro Steroid Production Bioassay**

No information submitted.

**K. In Vitro Germinal Vesicle Breakdown (GVBD) Bioassay**

No information submitted.

**XVII. Invertebrate Screens**

**A. Daphnia Reproduction (Life Cycle) Test**

**DESCRIPTION**

The objective of this test is to assess the effect of a chemical on the reproductive output of *Daphnia magna*. Information on growth is also obtained.

**REFERENCES**

EPA 850.1300, OECD 211, ASTM E 1193-93.

**DURATION**

21 days.

**EQUIPMENT**

Wet lab.

**STAFF SKILLS**

Basic.

**COST**

Moderate.

**AVAILABILITY**

Commercially available.

SENSITIVITY

Unknown.

SPECIFICITY

Apical for invertebrate endocrine related effects, unknown as far as vertebrates are concerned.

STANDARDIZATION

Yes.

RELATEDNESS

**B. Mysid Life Cycle Test**

DESCRIPTION

The objective of this test is to assess the survival, growth, and reproduction of mysids through a complete life cycle.

REFERENCES

EPA 850.1350, ASTM E 1191-90

DURATION

28 days.

EQUIPMENT

Wet lab, saltwater capability.

STAFF SKILLS

Basic.

COST

Moderate.

AVAILABILITY

Commercially available.

SENSITIVITY

Unknown.

SPECIFICITY

Apical for invertebrate endocrine related effects, unknown as far as vertebrates are concerned.

STANDARDIZATION

Yes.

RELATEDNESS

N/A

**Reptilian Screens**

**XVIII.**

**A. Vitellogenin Production In Adult Male Turtles**

TEST AND FUNCTION:

Vitellogenin production in adult male turtles (Red-eared slider, *Trachemys scripta*) and male frogs (*Xenopus laevis*); indicates estrogen receptor binding in the liver and/or estrogen receptor production.

**REFERENCES:**

Palmer, B.D. and S.K. Palmer, "Vitellogenin induction by xenobiotic estrogens in the Red-eared turtle and African clawed frog," *Environ. Health Perspectives*, 103 (suppl 4), 1995, pp. 19-25. (and others in the bibliography)

**DURATION:**

Injection daily for seven days, plasma collected on day 14.

**EQUIPMENT:**

General lab space for animals, equipment for ELISA.

**STAFF SKILLS:**

ELISA.

**COST:**

Not high; turtles and frogs purchased.

**AVAILABILITY:**

Animals easily obtainable.

**SENSITIVITY:**

Sliders more sensitive than frogs when comparing DDT-exposed to estradiol controls, but frogs equally as sensitive as sliders when comparing the absolute of vitellogenin produced in response to DDT.

**SPECIFICITY:**

Estrogen-dependant response.

**STANDARDIZATION:**

Good potential, depends on common source of antibody .

**OTHER:**

Exact same protocol used for amphibians; possibly useful as E- procedure with co-administration of estrogen.

**B. Sex Determination in Turtles****TEST AND FUNCTION:**

Sex determination in turtles; the red-eared slider (*Trachemys scripta*) and snapping turtles (*Chelydra serpentina*) have been used; responses measured are the morphology of the gonads and genital ducts and the histology of the gonads.

**REFERENCES:**

Bergeron, J.M., D. Crews, and J.A. McLaughlin, "PCBs as environmental estrogens: Turtle sex determination as biomarker of environmental contamination," *Environ Health Perspectives*, 102, 1994, pp. 780-781.

Crews, D., J.M. Bergeron, and J.A. McLaughlin, "The role of estrogen in turtle sex determination and the effects of PCBs," *Environ Health Perspectives* 103 (suppl 7), 1994, pp. 73-77.

Wibbles, T. and D. Crews, "Steroid-induced sex determination at incubation temperatures producing mixed sex ratios in a turtle with TSD," *General and Comparative Endocrinology* 100, 1995, pp. 53-60.

DURATION:

Rather long ~12 weeks to hatch and collection of samples.

EQUIPMENT:

General lab facilities.

STAFF SKILLS:

Tissue handling.

AVAILABILITY:

Eggs of *T. scripta* are commercially available.

SENSITIVITY:

Incubation temperature is crucial and needs some discussion for development of the best conditions for testing; there is greater *physiological* sensitivity to steroids at incubation temperatures that produce mixed sexes from the clutch, but greater *statistical* sensitivity when clutch is incubated at male producing temperatures.

SPECIFICITY:

An estrogen dependant alteration of male gonads.

STANDARDIZATION:

? Some subjectivity in the morphological and histological analysis.

OTHER:

The red-eared slider (*Trachemys scripta*) has been studied most extensively; are other species more appropriate or available? Again, possibly useful as a E antagonist procedure with co-administered estrogen at male temperatures, OR with EDC alone at female temperatures. This needs development, but would be worth the effort as so much of the basics are known in this system.